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Effects of Newcastle disease virus vaccine antibodies on the shedding and transmission of challenge viruses

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ABSTRACT

Different genotypes of avian paramyxovirus serotype-1 virus (APMV-1) circulate in many parts of the world. Traditionally, Newcastle disease virus (NDV) is recognized as having two major divisions represented by classes I and II, with class II being further divided into sixteen genotypes. Although all NDV are members of APMV-1 and are of one serotype, antigenic and genetic diversity is observed between the different genotypes. Reports of vaccine failure from many countries and reports by our lab on the reduced ability of classical vaccines to significantly decrease viral replication and shedding have created renewed interest in developing vaccines formulated with genotypes homologous to the virulent NDV (vNDV) circulating in the field. We assessed how the amount and specificity of humoral antibodies induced by inactivated vaccines affected viral replication, clinical protection and evaluated how non-homologous (heterologous) antibody levels induced by live NDV vaccines relate to transmission of vNDV. In an experimental setting, all inactivated NDV vaccines protected birds from morbidity and mortality, but higher and more specific levels of antibodies were required to significantly decrease viral replication. It was possible to significantly decrease viral replication and shedding with high levels of antibodies and those levels could be more easily reached with vaccines formulated with NDV of the same genotype as the challenge viruses. However, when the levels of heterologous antibodies were sufficiently high, it was possible to prevent transmission. As the level of humoral antibodies increase in vaccinated birds, the number of infected birds and the amount of vNDV shed decreased. Thus, in an experimental setting the effective levels of humoral antibodies could be increased by (1) increasing the homology of the vaccine to the challenge virus, or (2) allowing optimal time for the development of the immune response.
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1. Introduction

Infection of birds with virulent strains of Newcastle disease virus (NDV) causes one of the most important infectious diseases of poultry, Newcastle disease (ND), which is found worldwide and leads to economic losses from mortality and condemnation of carcasses. In 2010, seventy countries reported ND in domestic species to the World Organization for Animal Health (OIE) (www.oie.int.wahis/public.php?page=disease_status_lists) and many countries have endemic NDV, with outbreaks occurring year after year. Also known as avian paramyxovirus serotype-1 (APMV-1) virus, NDV is a member of the genus Avulavirus in the *Paramyxoviridae* family (Mayo, 2002a,b).

Antigenic similarity is shared among all NDV strains and isolates will cross-protect against challenge with any other NDV isolate. It is this immunological stimulation that serves as the basis of vaccination with live low virulent NDV (loNDV) to protect against virulent NDV (vNDV). Genetically, ND viruses are diverse and sixteen different genotypes have been already described (Courtney et al., 2012; Diel et al., 2012). Early studies have shown antigenic differences between strains of NDV using virus neutralization assays, hemagglutination inhibition (HI) assays with monoclonal antibodies, and by evaluating sequences of neutralizing epitopes (Panshin et al., 2002; Russell and Alexander, 1983; Schloer et al., 1975). The antigenicity of classes and genotypes can also be differentiated by cross HI assays, which correlate to differences in vaccine protection as measured by virus shedding after challenge (Gu et al., 2011; Li et al., 2010; Miller et al., 2007, 2009). While information regarding the avian immune response to NDV is limited, both antibodies and cell-mediated immunity (CMI) play a role in protection and clearance of NDV following infection (Reynolds and Maraqa, 2000a,b). Antibodies can be detected against NDV approximately 6–10 days post infection, while

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stimulation of antigen specific cytotoxic T-cells (CTLs) generally require about 7–10 days. Because the mean death time following infection with vNDV is 2–6 days, the presence of preexisting antibodies prior to infection appear to be most critical to protection from clinical disease (Kapczynski and King, 2005a). Antibodies produced against the hemagglutinin (HN) and fusion (F) trans-membrane surface glycoproteins are able to neutralize NDV upon subsequent infection (Boursnell et al., 1990a,b; Edbauer et al., 1990). In contrast, CTLs help clear from the host cells that are already infected and cannot stop disease progression. Since the pathology for loNDV is less than vNDV, the existence of preexisting immunity is not as critical to inhibit disease, and makes them excellent vaccine candidates.

Infection of chickens with vNDV results in rapid death of immunologically naïve birds, and thus the contribution of cell-mediated immunity is likely negligible since most birds are dead by 5–10 days post inoculation (Kapczynski and King, 2005; Kapczynski and Tumpey, 2003; Reynolds and Maraqa, 2000b). In contrast, infection with loNDV strains in immunologically naïve birds results in a limited, local infection, in which both humoral antibodies and antigen specific T-cells are generated. Clinical signs of infection are generally not observed in limited infections with loNDV, whereas the presence of secondary pathogens and/or immunosuppression can exacerbate clinical disease. In addition, mucosal immunoglobulin A (IgA) is produced in the respiratory tract and intestinal tract of chickens (Al-Garib et al., 2003a,b). Immunoglobulin G (IgG) can also be detected on mucosal surfaces and is believed to contribute to the overall local immunity as well (Chimeno Zoth et al., 2008). This mucosal antibody stimulation appears to aid in reduction of viral shedding, and will further aid in reducing viral infection following secondary exposure to NDV.

Antibodies to the HN and F glycoprotein of NDV are critical for virus neutralization and thus protection from vNDV (Reynolds and Maraqa, 2000a). Antibodies against the HN are responsible for blocking viral attachment, while antibodies against the F glycoprotein can inhibit viral fusion with the host cell membrane. Interestingly, even low levels of antibodies can provide protection of chickens against vNDV challenge (Gough and Allan, 1973).

Interest in the amount of vNDV shed into the environment by vaccinated birds has arisen as a potential indicator of vaccine efficacy (Miller et al., 2007, 2009). The ND experiments have shown that by using vaccines formulated with a NDV with the same (homologous) genotype of the vNDV challenge virus, for both genotype II and genotype V NDV isolates, it is possible to decrease not only the number of birds shedding vNDV, but also the amount of vNDV shed from individual birds by evaluating oropharyngeal and cloacal swab material (Miller et al., 2007, 2009). However, in those studies, the amount of virus shed from the birds vaccinated with vaccines heterologous to the genotype of the challenge virus was also decreased, but at lower amounts.

There is considerable controversy regarding the issue of vaccine failure on NDV control. Some argue that vaccine failure is mainly caused by inadequate application (Dortmans et al., 2012). However, others have suggested that vaccines formulated with genotypes homologous to the genotype of the challenge virus that reduce viral shedding should be a critical component of disease control (Hu et al., 2011). It is unknown if the use of higher doses of classical vaccines, which should induce higher antibody levels, would be sufficient to prevent ND caused by vNDV from genotypes more distant from vaccine strains, or which genotypes are more likely to fail vaccination with classical vaccines formulated with genotypes I and II NDV strains. In addition, it remains to be determined whether these older vaccines can significantly reduce viral shedding from challenge with newer isolates. In the present study, we vaccinated birds with a live LaSota vaccine and then challenged them with the heterologous vNDV (CA/2002) (defined as a virus of a different genotype) at different days post-vaccination (PV) to evaluate the amount of virus shed from each group and to subsequently determine how successful that amount of virus was transmitted to other birds. In addition, we further examined the seroconversion of chickens vaccinated with different genotypes of inactivated NDV and challenged with homologous and heterologous genotypes of vNDV to determine vaccine efficacy and humoral immunity on viral shedding. Our data indicate that vaccination with NDV vaccines formulated with antigens homologous (of the same genotype) of the challenge virus significantly reduces shedding compared to heterologous antigen, and that a correlation exists between antibody response after challenge with transmission potential to susceptible cohorts.

2. Materials and methods

2.1. Viruses

Working stocks of virus isolates used were obtained from the SEPRL repository and include US/LaSota/1946 (LaSota), gamefowl/USA (California)/212676/2002 (CA/2002), poultry/Peru/1918-13/2008 (Peru), Malaysia/1041/2008 (Malaysia) and Zoomat/Mexico (Chiapas)/2010 (Mexico). All viruses were propagated in 9–11 days old SPF embryos by chorioallantoic sac inoculation (Alexander and Swayne, 1998). The virulent CA/2002 strain (genotype V) was isolated as the etiological agent responsible for the last outbreak of ND in the US. The widely used LaSota vaccine (genotype II) was compared to recent vNDV viruses from Malaysia (genotype VIId), Mexico (genotype V) and Peru (genotype XII), a novel and highly divergent genotype related to recent African and Asian viruses in vaccine efficacy studies (Diel et al., 2012). Pools of infective allantoic fluid were clarified via centrifugation at 1000*g* for 15 min. Infectivity titers of the pools were determined by titration in SPF embryos prior to being stored at −70° C for use as live vaccine virus using hemagglutination (HA) assays.

2.2. Chickens and vaccine preparation

For all experiments, mixed-sex SPF chickens were obtained from the Southeast Poultry Research Laboratory SPF flocks and transferred to a BSL2 or BSL3E facility for vaccination and a BSL3E facility for challenge. Birds were maintained in Horsfal isolation units with feed and water administered *ad libitum*. In experiment II (below), four experimental NDV inactivated vaccines were produced with the LaSota, Mexico, Peru and Malaysia isolates following growth in SPF eggs and harvesting of allantoic fluid. Oil emulsion-adjuvanted vaccines were prepared as described by Stone et al. (1978). Following BPL-inactivation (Miller et al., 2007) of allantoic fluid, each vaccine virus was diluted to provide a concentration of approximately $10^{9.5}$ EID_{50}/dose (0.5 ml). Sham vaccine was prepared as above with normal allantoic fluid harvested from SPF embryos.

2.3. Challenge experiment I

Birds were vaccinated with a live vaccine and challenged with virulent CA/2002 at 3, 10 or 21 days post vaccination (Table 1). Birds were given 100 μl of a live LaSota vaccine ($10^{6.5}$ EID_{50}) with half the dose given onto the right eye and the other half into the choanal cleft. Sham vaccines for the controls and non-vaccinated contacts consisted of sterile BHI and were given as described above. The birds were challenged with the selected CA/2002 virus with the specified (Table 1) mean 50% embryo infectious dose (EID_{50}) of 100 μl per bird, half administered in 50 μl into the right eye and half in 50 μl into the choana. At 48 h post challenge, the 10

Table 1
Experimental design for experiment I demonstrating the difference in the time of vaccination and challenge with vNDV CA/02 and the number of birds in each of the groups.

Group[a]		# Birds/Group
1-Sham/C	No vaccine/challenged	10
1-Con/Vax	Vaccine/contact[b]	5
1-Con/NV	No vaccine/contact	5
2–3dPV/C	Challenged 3dPV	10
2-Con/3dPV	Vaccine/contact	5
2-Con/NV	No vaccine/contact	5
3–10dPV/C	Challenged 10dPV	10
3-Con/10dPV	Vaccine/contact	5
3-Con/NV	No vaccine/contact	5
4–21dPV/C	Challenged 21dPV	10
4-Con/21dPV	Vaccine/contact	5
4-Con/NV	No vaccine/contact	5

[a] C = Challenged; Con = Contact birds; dPV = days post vaccination; NV = Non-vaccinated.
[b] Contact birds mixed with challenged birds at 2 days post-challenge.

birds for each group were bled, swabbed (oropharyngeal and cloacal) and half were moved into a clean isolator with 5 vaccinated contact (non-challenged birds) and 5 were moved into a clean isolator with 5 non-vaccinated contact (non-challenged) birds. The floors of the isolators were covered with paper to prevent fecal matter from falling through the grated floor to allow access to the contaminated feces, as it would be in a commercial setting where birds are raised on the floor. Both the challenged and contact birds were bled before vaccination and before challenge or contact with challenged birds to evaluate antibody levels. 2 days and 4 days after the challenged birds and contact birds were placed into the same isolator, oropharyngeal and cloacal swabs were collected from the challenged and contact birds into 1.5 ml of BHI broth with a final concentration of gentamicin (200 μg/ml), penicillin G (2000 units/ml), and amphotericin B (4 μg/ml). Birds were monitored daily for clinical signs and death through at least day 21 post-challenge when they were sedated, bled and euthanized. Moribund chickens were euthanized with intravenous sodium pentobarbital at a dose of 100 mg/kg and counted as dead on the next day. This experiment was performed two times.

2.4. Challenge experiment II

One hundred fifty, 4 weeks old SPF chicks were randomly divided in groups of 10 animals in BSL2 and vaccinated subcutaneous with a single dose of each vaccine virus on the neck. The inactivated vaccine strains included LaSota (genotype II), Mexico (genotype V), Malaysia (genotype VIId) and Peru (genotype XII). Serum was collected and analyzed prior to vaccination to ensure the SPF flock was indeed NDV negative. A group of 10 birds remained unvaccinated to serve as the sham-vaccinated control group for each challenge virus. Clinical signs of NDV were monitored in all groups daily for any vaccine reactions, which would be unlikely for inactivated vaccines. At 3 weeks post vaccination prior to challenge, serum samples were collected for all animals and group of birds were moved to BSL3 facility and challenged with homologous and heterologous vNDV of different genotypes. The serum samples were evaluated for NDV antibodies by hemagglutination inhibition (HI) (Miller et al., 2007). Each group of chickens was challenged with a different strain of vNDV corresponding to the selected genotypes. Chickens were monitored for clinical signs daily and oral and cloacal swabs were collected at day 4 post-challenge to evaluate the amount of challenge virus shed from each bird. Morbidity and mortality were followed for 2 weeks post-challenge. At the termination of the trial, serum was collected to measure post-infection NDV antibody titers for all survivors. Humoral immunity was evaluated by the HI test on serial 2-fold dilutions.

2.5. Serology

Serum was obtained pre and post challenge from all birds and tested by hemagglutination inhibition (HI) assay. The HI assay was performed using inactivated NDV antigen according to standard procedures with 4 HAU virus/antigen in 0.025 ml (OIE, 2012). Titers were calculated as the highest reciprocal serum dilution providing complete hemagglutination inhibition. Serum titers of 1:8 (2^3) or lower were considered negative for antibodies against NDV.

2.6. Virus shedding

Virus isolation (VI) was performed to identify virus shedding conducted on oral and cloacal swabs as previously described (Miller et al., 2007). All VI-positive swabs were titrated in 9–11 days old SPF ECE as described (Alexander and Senne, 2008). Virus titers were calculated using the Spearman–Kärber method (Kaerber, 1931) and were reported as mean embryo infectious dose (EID_{50}/0.1 ml) on a Log 10 scale.

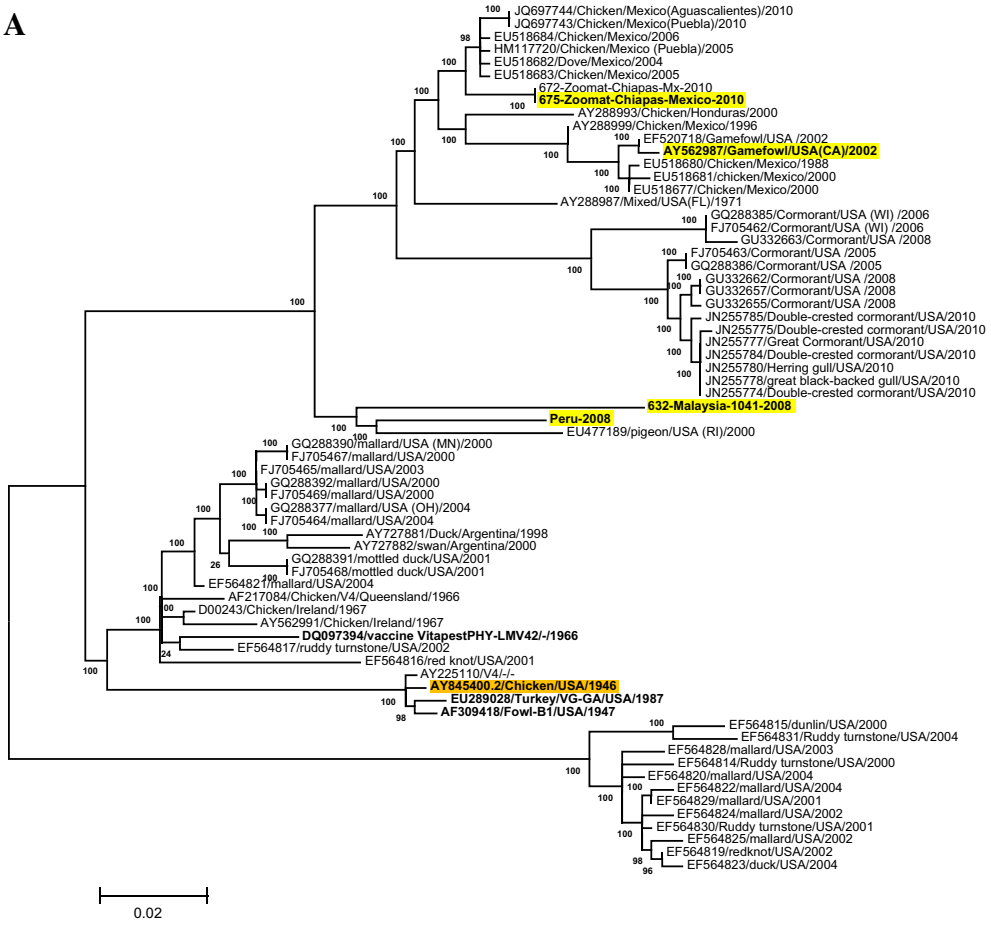
2.7. RNA extraction and sequencing

RNA extraction and sequencing was done as previously described (Kim et al., 2007). Briefly Total RNA was extracted by mixing 250 μl of allantoic fluid with 750 μl of Trizol LS reagent (Invitrogen, Carlsbad, CA), following manufacturer's instructions. The fusion (F) gene was amplified by reverse transcription and polymerase chain reaction (RT-PCR) using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). Amplicons were sequenced with fluorescence dideoxynucleotide terminators in an ABI 3700 automated sequencer (Applied Biosystems, Fosters City, CA). Assembly and editing of sequencing data was performed using the DNASTAR Laser Gene software package, version 10.0. Sequences of Malaysia and Mexico isolates have been sent to GenBank and the accession numbers are KC808511, KF011206, and KF011207.

2.8. Phylogenetics

Phylogenetic analysis was done by comparing the fusion protein. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992) (Fig. 1A). The tree with the highest log likelihood (−4489.0128) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories (+G, parameter = 0.7050)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 29.3614% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 68 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 550 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

A



B

	LaSota	Mexico	CA02	Peru	Malaysia
LaSota		88.6	87.7	87.9	88.1
Mexico	12.4		94.6	93.3	91.9
CA02	13.5	10.5		90.8	89.9
Peru	13.2	7.0	9.9		92.8
Malaysia	12.7	8.3	10.5	7.2	

C

	LaSota	Mexico	CA02	Peru	Malaysia
LaSota		88.1	89.0	86.7	86.9
Mexico	12.8		94.8	89.7	89.7
CA02	11.8	5.5		90.6	90.0
Peru	14.5	11.1	10.1		90.7
Malaysia	14.3	11.1	10.7	9.9	

Fig. 1. (A) Molecular Phylogenetic analysis of Newcastle Disease isolates by Maximum Likelihood method. Analysis of the full fusion protein was performed as described in the Section 2. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 68 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 550 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [2]. Virulent viruses utilized in vaccination experiment are highlighted in yellow (virulent viruses) and vaccine viruses are highlighted in orange. (B) Fusion protein amino acid differences between the LaSota and B1 vaccine viruses compared to selected vaccine and virulent challenge viruses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.9. Statistical analysis

Kaplan–Meier survival curves were generated with Prism 5 (GraphPad Co., San Diego, CA). The Mantel–Cox log-rank test was used to compare survival curves between two experimental groups (Prism 5). Statistical differences in mean and standard error between mean HI titers were analyzed using Tukey one-way ANOVA (Prism 5). Lower case letters indicate statistical significance between compared groups. All statistical tests were performed using $P < 0.05$.

3. Results

3.1. Phylogenetics

All isolates used in these studies were compared using phylogenetics. Representative NDV strains of the most recent circulating genotypes were selected for use as antigen in the inactivated vaccine experiment (Fig. 1A). Selection was based on phylogenetic distance and amino acid differences based on the NDV F gene. The Peru (genotype XII) and Malaysia viruses (genotype VIId) represent isolates from recent outbreaks in South America and Asia, and the Mexico and CA/02 are representative of isolates of genotype V circulating in North America.

Amino acid similarities for the key proteins involved in neutralizing NDV was determined by comparing the F and HN proteins of field isolates with the LaSota vaccine viruses using ClustalW with a PAM250 matrix. The amino similarity at the fusion protein (upper triangle) and percent divergence (lower triangle are represented) are presented in Fig. 1B. In Fig. 1C a similar representation of the comparison for the HN proteins is presented. The similarity between LaSota vaccine and circulating viruses varied between 87.7% and 88.6% for the F protein and between 86.7% and 89.0% for the HN protein. Among current circulating viruses the similarities varied between 89.9% and 94.6% for the F proteins, and between 89.7% and 94.8% for the HN proteins. This comparison confirms that the LaSota vaccine virus is more distantly related to circulating viruses than these are among themselves.

3.2. Protection from challenge in experiment I

The purpose of this study was to determine the role of the immune response to a vaccine formulated with a NDV of a different genotype than the genotype of the challenge virus on transmissibility of vNDV in chickens infected at different times post-vaccination (Table 1). All of the non-vaccinated contact birds (Con/NV in Groups 1, 2 and 3), which were placed with birds that were challenged before 21 days post-vaccination (PV), died between 4 and 19 days of contact (Fig. 2). Although all non vaccinated birds placed into direct contact with directly challenge birds shed virus at day 2 post-contact, it is interesting to note that the level of shedding in the contact groups (Groups 1–4 Con/NV) decreased as the time between vaccination and direct challenge increased from no vaccine to 3, 10 and 21 days between vaccination and challenge (Table 2). All of the vaccinated contact birds (Group 1-Con/3dPV, Group 2–10dPV, and Group 3–21dPV) survived contact with the challenged

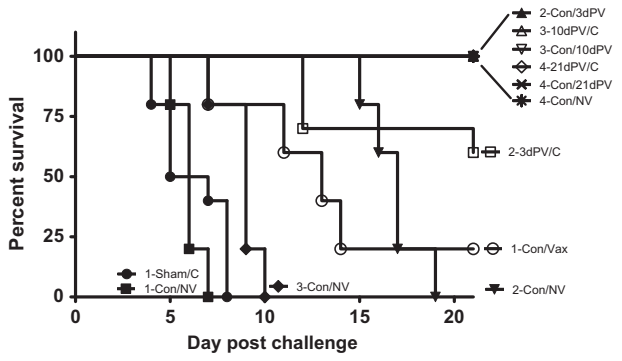


Fig. 2. Duration of protection and transmission following vaccination of chickens with LaSota virus to lethal vNDV (CA/02) challenge. Birds vaccinated at various intervals between challenge (0, 3, 10 and 21 days post-vaccination) were tested for protection and contact (Con) shedding to vaccinated (Vax) and non-vaccinated (NV) cohorts. The percent survival versus day post challenge was monitored for 21 days following challenge or contact.

birds. However, 75% of the birds in Group 1 Con/Vax did show moderate to severe torticollis demonstrating transmission to this group, and were humanly euthanized. The overall effect of increasing the time between vaccination and challenge resulted in variation in the mortality from 40% at 3d (3dPV/C), 0% at 10d (10dPV/C), and 0% at 21d (21dPV/C) ($P < 0.05$).

As the time between vaccination and challenge increased, the amount of virus shed from the challenged birds also decreased (Table 2). Additionally, with time there was only a small increase in the antibody titers of the pre-challenge serum to the CA/02 antigen (Table 2). The HI antibody levels were negative in all birds prior to challenge or contact with infected birds. The HI titers of vaccinated birds of group 3 (3–10dPV/C and 3-Con/10dPV) pre-challenge were 5.1 and 6.5 ($\log_2$), respectively (Table 2). The birds in this group that were directly challenged demonstrated increased HI titers (from 5.1 to 7.9), indicating virus replication, which was observed as virus shedding in oral and cloacal swab samples (Table 2). In contrast, no appreciable increase in HI titers [6.5–6.9 ($\log_2$)] was observed in the vaccinated contact birds placed in the same isolator with the directly challenged birds. No virus was recovered in oropharyngeal or cloacal swabs from these birds 2 days after these birds were mixed together with the vaccinated birds directly challenged. Similar results between vaccinated-challenged (21dPV) and contact vaccinated birds were observed in Group 4 (Table 2) in that no virus was recovered from the vaccinated contact birds 2 days after they were mixed with the vaccinated directly challenged birds.

It is important to note that contact non-vaccinated birds in this group did not become infected, shed virus or develop an antibody response. The results demonstrate that as the days between vaccination and challenge increase from 3 to 10 days or 21 days, the percentage of non-vaccinated contact birds infected decreases from 100%, 80% and 0%, respectively. As expected, when the LaSota vaccine antigen was used in the HI assay, the pre-challenge antibodies levels were found to be higher than the results obtained when the same sera was tested with the challenge antigen, CA02 (Table 2).

3.3. Protection from challenge in experiment II

The purpose of this study was to investigate effect of inactivated vaccine antigen on protection and shedding following homologous or heterologous challenge. With one exception (80% survival in Mexico NDV vaccine against Malaysia NDV challenge), survival was 100% for vaccinated animals and mortality was 100% for sham-vaccinated animals; however, not all challenge viruses exhibited the same virulence (Fig. 3). For example, animals challenged with the Mexico or the Peru viruses demonstrated a mean death time (MDT) of 3.5 days and 5 days, respectively. Unexpectedly, the Malaysia NDV strain caused a slow and uncharacteristic mortality with MDT of 10 days. In terms of protection only the vaccine formulated with the Mexican antigen did not induce 100% survival against the Malaysian challenge. The uncharacteristic behavior of the Malaysia virus is surprising and merits further study.

All animals were negative for NDV HI antibodies at the beginning of the experiment and all vaccines induced a significant immune response (Table 3). HI antibody titers for all vaccine groups were determined to be between 6 and 7 ($\log_2$) with the LaSota antigen, but the titers increased when the antigen used in the vaccine was used in the HI assay. In the LaSota-vaccinated animals, a rise in antibody titers was observed after challenge with each isolate, suggesting that challenge virus replication was not completely prevented by the vaccination protocol. This group demonstrated the highest average change between pre- and post-challenge titers than the other groups. In the animals vaccinated with the Malaysia

Table 2
Serology and virus shedding in Experiment I. Pre- and post-challenge HI antibody titers (log_2) to the CA/02 virus are shown. Viral titers (log_{10}) expressed as mean embryo infectious doses per 0.1 ml from oral and cloacal swabs taken at day 2 post-challenge for the challenged birds (C) or post-contact for the contact birds (Con).

Group[a]	Pre/Post Challenge HI titer[b]		Viral load in swab samples taken at day 2 post-challenge or post contact with challenged birds	
	LaSota Antigen	CA02 Antigen	Oral	Cloacal
1-Sham/C	<2/NS	<2/NS	6.6	5.4
1-Con[c]/Vax	<2/NS	<2/NS	5.1	4.0
1-Con/NV	<2/NS	<2/NS	5.0	4.1
2–3dPV/C	<2/9.7	<2/9.7	3.7	4.2
2-Con/3dPV	<2/6.0	<2/7.1	1.7	1.1
2-Con/NV	<2/NS	<2/NS	4.5	3.5
3–10dPV/C	8.3/7.5	5.1/7.9	2.3	0.5
3-Con/10dPV	8.1/5.2	6.5/6.9	0	0
3-Con/NV	<2/NS	<2/NS	2.9	1.5
4–21dPV/C	6.8/9.1	5.4/8.2	0.6	0
4-Con/21dPV	6.7/6.0	5.2/6.0	0	0
4-Con/NV	<2/<2	<2/2	0	0

[a] C = Challenged; Con = Contact birds; dPV = days post vaccination; NV = Non-vaccinated; Vax = vaccinated at different times prior to introduction of infected birds.
[b] Birds vaccinated with LaSota vaccine with titers obtained with the LaSota and CA/02 antigens.
[c] Contact birds mixed with challenged birds at 2 days post-challenge.

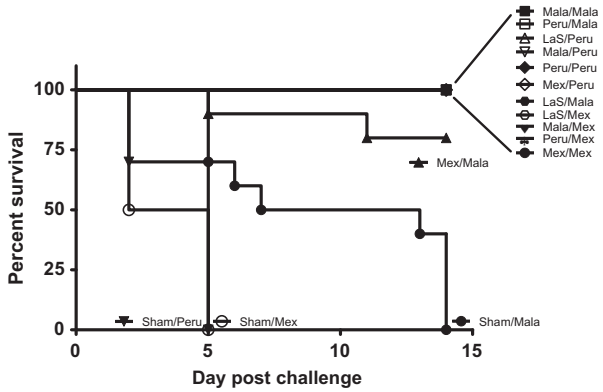


Fig. 3. Homologous versus heterologous vaccination and challenge against recent vNDV isolates. Birds were vaccinated with various vaccine antigens (LaSota, Malaysia, Mexico, or Peru) and challenged with homologous or heterologous vNDV (Malaysia, Mexico, or Peru). Three weeks post-vaccination birds were challenged with lethal vNDV and the percent survival was monitored for 14 days.

NDV strain, the pre-challenge titers were 9.2, 6.4 and 6.8 for the batches challenged with Malaysia, Mexico, and Peru, respectively. In the groups of animals vaccinated with the Mexico NDV strain, the homologous pre-challenge titers were 7.0, 8.6, and 7.0 for animals to be challenged with Malaysia, Mexico, and Peru, respectively. No significant increase in HI titers was observed after challenges with Peru or Mexico; however, a significant increase was observed in the animals infected with the Malaysia NDV isolate. The lack of a significant increase in HI titers observed after challenges with Peru and Mexico suggest that those vaccines likely prevented virus replication after challenge with the homologous NDV strain. However, the homologous challenge with the Malaysia vaccinated birds produced a significant increase in titers. In the animals vaccinated with Peru, the pre-challenge titers were 5.5, 5.3 and 6.2, for the animals challenged with Malaysia, Mexico, and Peru, respectively. This was the only group in which pre-challenge titers were not the highest against the homologous virus. In all cases, the antibody titers increased post-challenge, again suggesting that those vaccines did allow some viral replication.

Virus shedding in oropharyngeal swabs was significantly reduced in homologous vaccinated-challenged animals compared to sham-vaccinated (control) birds for two of the three challenge viruses (Fig. 4). A 4-log reduction in titer was observed in the Malaysia-vaccinated/Malaysia challenged group compared to sham-vaccinated birds, whereas the Malaysia-vaccinated/Malaysia-challenged group demonstrated an approximate 1.5-fold reduction compared to heterologous-vaccinated/Malaysia-challenged groups. A similar pattern was observed with the Mexico-vaccinated/Mexican-challenged birds, as well. Homologous vaccinated birds demonstrated a 5-log reduction from sham-vaccinated birds, and a 2-log reduction from heterologous-vaccinated/Mexico-challenged birds. In contrast, all vaccinated birds (both homologous and heterologous vaccines) challenged with the Peru virus demonstrated a similar level of reduced shedding, approximately 2-fold compared to the sham-vaccinated/Peru-challenged birds, with no significant difference observed between the vaccine groups. All vaccines significantly reduced shedding in cloacal swabs compared to sham-vaccinated birds but no significant differences were obtained when comparing the amount of virus shed between the different vaccines (data not shown).

3.4. Correlation of humoral response and shedding

To test the effects of the humoral immune response to viral shedding in surviving birds, we correlated the difference in HI

Table 3
Pre- and post-challenge HI antibody titers (log_2) to the homologous and heterologous antigens used in Experiment II. Titers homologous between the vaccine and challenge virus are bolded. The post challenge titers are in parenthesis to the right of the pre-challenge antibody titers.

Serum vaccine groups	HI Antigen						
	LaSota	Malaysia	Difference	Mexico	Difference	Peru	Difference
LaSota	**6.6**	5.5 (8.6)	3.06	5.7 (8.1)	2.37	6.5 (7.9)	1.48
Malaysia	6.0	**9.2 (10)**	**0.83**	6.4 (8)	1.56	6.8 (8.8)	2.00
Mexico	6.8	7.0 (10.3)	3.20	**8.6 (8.6)**	**0.00**	7.0 (7.9)	0.90
Peru	6.1	5.5 (7.4)	1.9	5.3 (7.3)	2.0	**6.2 (8.3)**	**2.06**

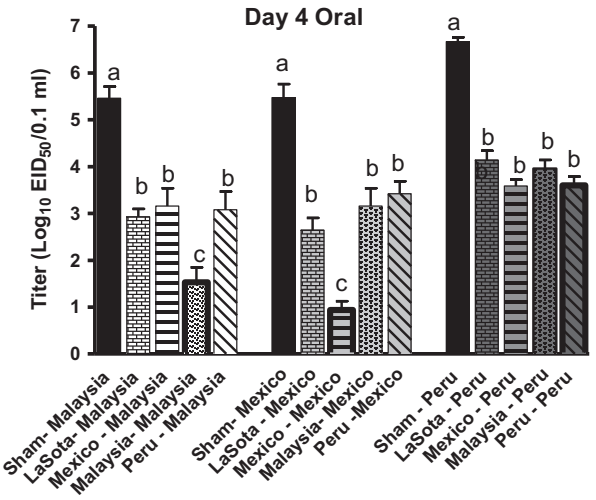


Fig. 4. Viral titers obtained from oral swabs on day 4 post-challenge in experiment II. Birds were vaccinated at 2 weeks of age and challenged with homologous or heterologous virus at 5 weeks of age.

titers (post-challenge minus pre-challenge) to viral shedding titers. In experiment I, a strong correlation (0.78) was observed between groups of surviving birds when viral shedding titers were compared to antibody response (Fig. 5A). In these comparisons, contact birds from groups 3-Con/10dPV, 4-Con/NV, and 4-Con/21dPV did not shed virus and had no change in HI response. The relationship was confirmed with birds shedding various levels of virus (between 0.6 and 3.6 $\log_{10}$ EID_{50}/0.1 ml), and a relative gain in antibody response. Of note is the observation that when challenge was applied early after vaccination, a higher level of shedding was observed (e.g. Group 2–3dPV/C) than groups challenged later after vaccination (e.g. 3–10dPV/c or 4–21dPV/C). In the second replication of this experiment one bird from group 4–21dPV/C was infected and did have an anamnestic response in HI titer. In experiment II a linear correlation (0.691) was also observed between the surviving groups (Fig. 5B). As expected, groups of vaccinated birds challenged with homologous vNDV exhibited low amounts of virus shedding, which resulted in little to no change in HI titer. In contrast, vaccinated birds challenged with heterologous virus demonstrated greater changes in HI titers and higher levels of virus shedding.

4. Discussion

The study of the transmission of Newcastle disease virus has been and continues to be a relevant topic for the poultry industry as it is important for understanding not only how to prevent the spread of vNDV, but also how to enhance protection of mass-vaccinated flocks (Delay, 1948; Estola et al., 1979; Li et al., 2009). In addition to airborne transmission, NDV can spread though direct contact with infected birds, contaminated poultry products consumed by other birds, people with contaminated clothes or shoes, equipment or vaccines (Alexander, 1988). Infection from ingestion of contaminated feed (Alexander et al., 1984), water (Saber et al., 1978) and flies found in a poultry house (Chakrabarti et al., 2007) may also occur. It is possible, and likely, that more than one of the aforementioned factors would contribute to the spread and transmission of NDV among birds depending on each situation.

Like most vaccines, NDV vaccines do not prevent vaccinated animals from becoming infected with a vNDV and subsequently shedding the virus (Kapczynski and King, 2005). However, most vaccines will significantly decrease the amount of virus shed in saliva and feces compared to non-vaccinated birds (Miller et al., 2009). The amount shed will depend on the immunity of the host, the host species infected, the amount and virulence of the challenge virus, the dose and type of ND vaccine and the time between vaccination and challenge. While the amount will vary depending on the NDV isolate and the host species, it is thought that (under experimental conditions) each host would need to receive between 10^3 and 10^4 EID_{50} of virus to become infected with NDV (Alexander et al., 1999; King, 1996). With these and previous experiments (Miller et al., 2009) using LaSota vaccine and CA/2002 challenge virus the question remained as to if the amount of virus shed would be enough to infect other naïve and vaccinated birds.

Unfortunately, due to the requirements of working with vNDV in an isolator with a HEPA filter and in a room with negative pressure, and necessary air exchanges, our experimental conditions are likely to be dissimilar than those found in the field. However, even with 12 air exchanges per hour, which would significantly decrease the amount of available aerosolized virus, we were able to get transmission to almost all of the non-vaccinated contact birds when they were placed into isolators with sub-optimally vaccinated or non-vaccinated challenged birds. In this experimental setting, with SPF birds, the LaSota vaccine provided 100% protection against clinical disease and death at 10 days post vaccination challenge. Interestingly, none of the non-vaccinated contact birds became infected when mixed with the birds challenged 21 days PV. This highlights the importance of (1) having enough time between vaccination and challenge to develop sufficient immunity and (2) the importance of flock (herd) immunity in the protection conferred with vaccines (van Boven et al., 2008).

A similar result was observed with challenges at 10 days PV. The vaccinated contact birds were not infected, but the naïve non-vaccinated birds were infected. The environmental contamination that exists in an outbreak setting and/or with any vaccinated population should be taken into consideration for Newcastle disease control. This is especially true of countries with endemic vNDV. For each country, it is important to know the characteristics and virulence of the vNDV circulating and which vaccines provide the largest decrease in vNDV shed so that less vNDV is put into the environment. While all NDV isolates are of one serotype, some strains (antigens) that used to circulate are no longer found in some areas of the world (Miller et al., 2010). As demonstrated here, even small amounts of vNDV shed by birds vaccinated with the LaSota vaccine may pass to other vaccinated birds if flock immunity is low. However, when flock immunity increases, even low levels of antibody titers may be sufficient to prevent infection depending on the challenge dose. Another important point of this study is that the differences in immune responses observed between day 3, 10 and 21 seem to be very critical as a determinant of transmission, thus suggesting that even heterologous vaccines can prevent transmission if sufficient time is allowed for birds to mount a proper immune response. Furthermore, the long delay of 21 days to achieve suppression of transmission suggests that future studies on vaccination should focus on ways to accelerate speed of the immune response in addition to the use of homologous antigens.

Despite of the use of an equal amount of antigen in experiment II, not all inactivated vaccines produced the same levels of pre-challenge humoral antibodies. The factors involved in those different responses are currently unknown. The LaSota and Peru vaccines induced the lowest pre-challenge antibody levels. However, even for these vaccines there was, in most cases, 100% protection against mortality and clinical signs. Overall, the increase in antibody titers post-challenge for the LaSota and Peru vaccines indirectly suggests that these vaccines may be not be as effective in protecting against viral replication as they are of protecting against clinical signs, and perhaps that homology is important.

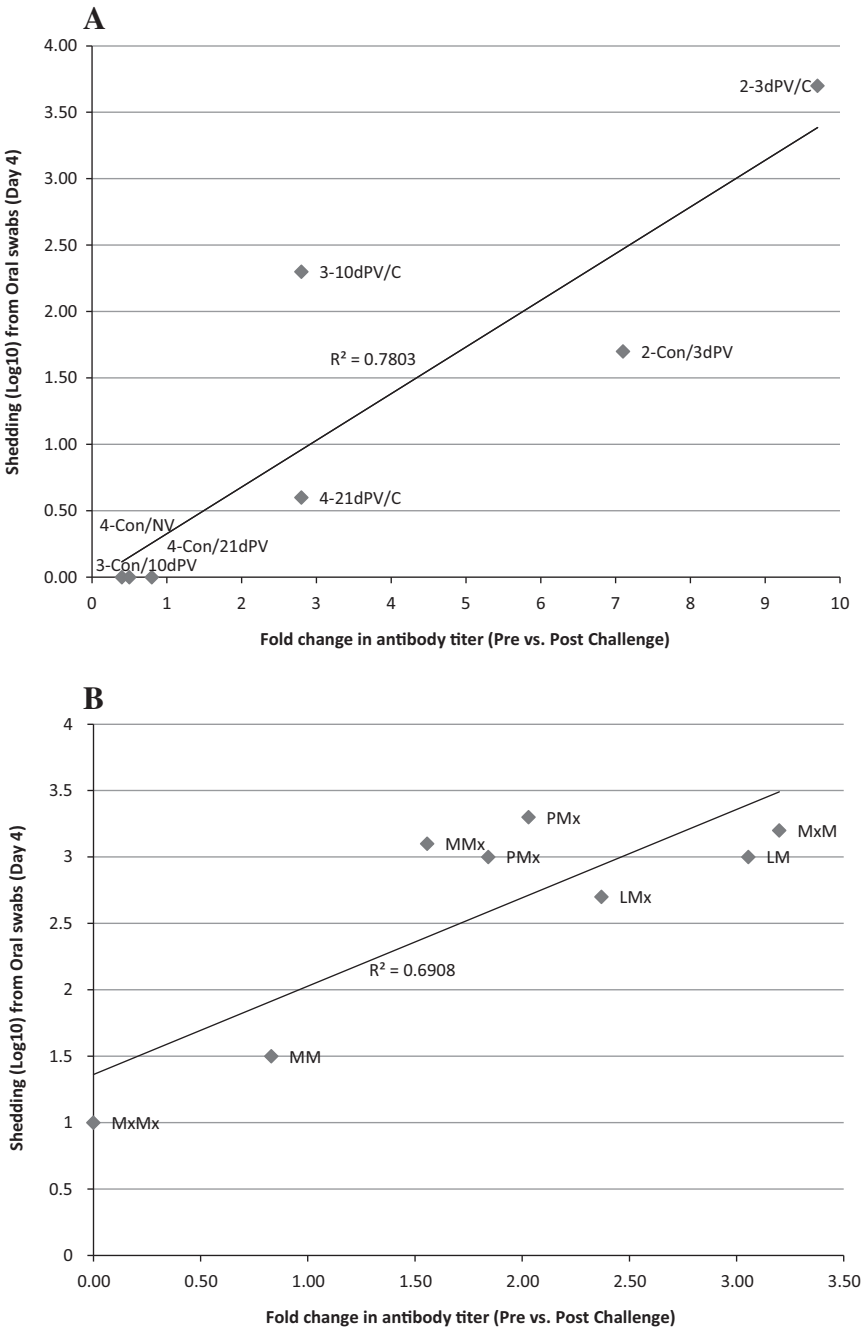


Fig. 5. Correlation between antibody response and viral shedding in Experiments I (A) and II (B). The change in HI antibody response between pre- and post-challenge was plotted against corresponding mean virus shedding for that group. The relative correlation between the data points is given by *R*-value. (B) Each point is represented by the vaccine antigen (first letter) and challenge virus (second letter) with L = LaSota, M = Malaysia, Mx = Mexico, P = Peru.

The Malaysia and Mexico antigens produced the highest pre-challenge antibody levels. Birds vaccinated with these antigens did not produce a significant increase in antibody titers after challenges, suggesting that a high threshold of humoral immunity can reduce viral replication, independent of genotype of the vaccine. These data are in agreement with recently published data for viruses of genotype VIId showing that a live heterologous LaSota vaccine was not effective in preventing viral replication at clinically protective doses (10^3 to 10^5) (Cornax et al., 2012). However, if the vaccine dose was sufficiently high (10^6 to 10^8 EID_{50}/bird) virus replication (measured indirectly as post challenges increases in antibody titers) was prevented. The surprisingly partial protection of Mexico against Malaysia, and the unusual behavior of the Malaysia viruses on the sham-vaccinated animals, suggests that further studies need to be done to characterize the Malaysian viruses.

Results in Fig. 1A–C confirm that observed genomic distances correlate with amino acid differences in the F and HN proteins. However, these differences in amino acid identity are large and a simple amino acid comparison is not likely to be a sufficient indicator of antigenic similarities, suggesting that the selection of vaccine antigens still will have to be done based on empirical cross protection studies with live animals. It is interesting to note that there seems to be an increased genetic variability in HN in comparison with the F protein; however, it is still too early to predict if any of those genes are going to be suitable predictors of cross protection. One would expect the antigens with the least HN genome

similarities (Fig. 1C) to produce the worst HI results (Table 3), but this only occurred for the birds vaccinated with the Mexican and Malaysian antigens.

Virulent NDV continues to be endemic in many countries around the world despite the application of billions of doses of live, inactivated, and recombinant NDV vaccines. Our studies indicate that matched vaccines to field isolates have potential to provide superior protection against transmission by reducing the magnitude of viral shedding. In the field, multiple factors may decrease the effectiveness of vaccination thus making the antibody specificity more important. Further investigation as to the best vaccine for individual situations, focusing not only on prevention of clinical disease and mortality, but also on decreasing the amount of virus shed from vaccinated birds is an important consideration in countries with endemic vNDV.

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